TRANSGENIC BIOSENSORS FOR ORGANOPHOSPHOROUS POISONING AND THE PROTECTIVE EFFICACY OF DEFENSE AGENTS

TOWARD AN ENGINEERED CHOLINESTERASE DECOY

HERMONA SOREQ, Ph.D.
Department of Biological Chemistry
The Life Sciences Institute, The Hebrew University
Jerusalem 91904, Israel

AIMS

We have successfully developed the transient transgenic *Xenopus* tadpole as a useful *in vitro* model in which to test potential defense agents (Ben Aziz-Aloya *et al.*, 1993). We are now directing attention to development of the defense agent itself. Our goal has been to develop an antidote for organophosphorus chemical agents (OPs), based on an engineered human butyryl-cholinesterase (hBuChE). Among the reasons for basing the development on hBuChE are:

1. It already offers limited protection, reacting with OPs and sparing acetylcholinesterase (hAChE) of the CNS and neuromuscular junctions.
2. As a serum enzyme, it will protect against systemic effects of OPs.
3. Variants are likely to be non-immunogenic, as there are no known autoimmune complications involving hBuChE, and the changes we wish to introduce in the enzyme are not on exposed surfaces.
4. Natural variations of the level of hBuChE activity are well-tolerated (Neville *et al.*, 1992).

How will we recognize the ideal variant when we meet it?
Our operational requirements for an improved hBuChE are that it:

1. be more sensitive to low levels of OPs,
2. react faster with OPs,
3. be reactivated faster by 2-PAM, and
4. react with a broad range of OPs.

GENERAL CONCEPT

What are the structural requirements of the ideal variant hBuChE? In what general region of the protein are the amino acid residues that interact with OPs? What specific residues are involved?
We know that OPs are hemi-substrates: like substrates they react with an active site residue (Ser^{198}), but unlike substrates, they are not quickly hydrolyzed. This fact confines attention to the active site region of the protein and to the long narrow gorge through which OPs must pass (FIG. 1).

To further narrow the number of residues we must consider, we made a chimeric protein, substituting a sequence of hAChE residues for the same sequence in hBuChE. These residues comprise some of the rim of the active site gorge, about half the residues lining the gorge, and several residues at the choline-binding site (FIG. 2). We surveyed a variety of OPs and other anti-

COMPONENT PART NOTICE

THIS PAPER IS A COMPONENT PART OF THE FOLLOWING COMPILATION REPORT:

TITLE: Proceedings of the Medical Defense Bioscience Review (1993) Held in Baltimore, Maryland on 10-13 May 1993. Volume 3.

TO ORDER THE COMPLETE COMPILATION REPORT, USE AD-A275 669.

THE COMPONENT PART IS PROVIDED HERE TO ALLOW USERS ACCESS TO INDIVIDUALLY AUTHORED SECTIONS OF PROCEEDING, ANNALS, SYMPOSIA, ETC. HOWEVER, THE COMPONENT SHOULD BE CONSIDERED WITHIN THE CONTEXT OF THE OVERALL COMPILATION REPORT AND NOT AS A STAND-ALONE TECHNICAL REPORT.

THE FOLLOWING COMPONENT PART NUMBERS COMPRISE THE COMPILATION REPORT:

AD#: AD-P008 853 AD#:

AD#: thru AD#:

AD#: AD-P008 900 AD#:

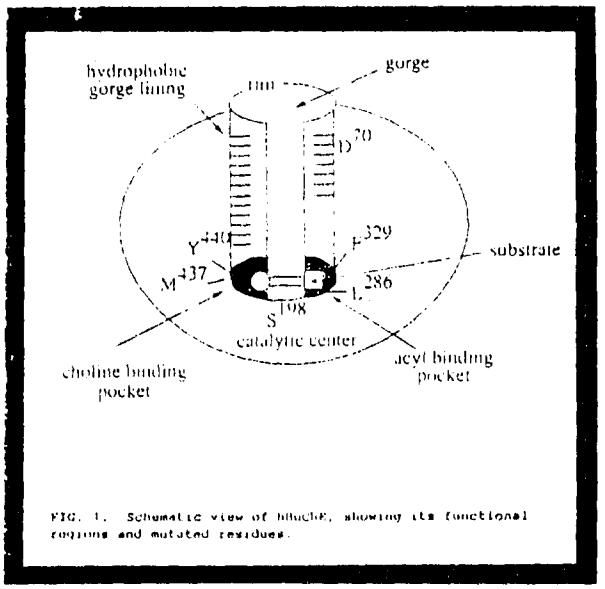

FIG. 1. Schematic view of hBuChE, showing its functional regions and mutated residues.

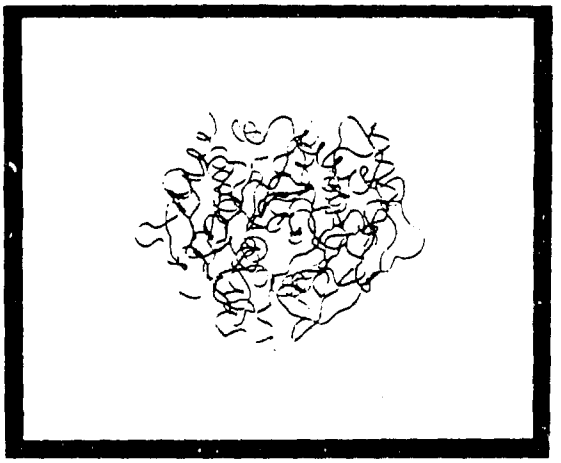

FIG. 2. Ribbon diagram of the hBuChE/hAChE chimera. The hBuChE portion is shown in magenta, and the replaced residues (58 through 133 of hAChE) are shown in blue.

cholinesterases by determining their IC_{50} values. The results (TABLE 1), and a comparison of the sequence of hBuChE with that of hAChE in this region (FIGS. 3, 4), direct attention to residues Asn^{68}, Asp^{70} and Gln^{119} in hBuChE and the equivalent residues Tyr^{72}, Asp^{74} and Tyr^{124} in hAChE.

By site-directed mutagenesis we made a series of single amino acid replacements at and near the active site (FIG. 5). The results (TABLE 2) eliminate from consideration Leu^{286}, Met^{435} and Ser^{198}, and focus attention on Phe^{329} and Tyr^{440}.

METHODOLOGY

Our aims and the process of selection require a very sensitive novel method of assaying variant hBuChEs:

1. We produce by molecular biological techniques small quantities of a large number of natural and engineered variants, and express them in *Xenopus* oocytes. After overnight incubation, activity of a single oocyte reaches about 5 nmol/min (wild-type, assayed against butyrylthiocholine, pH 7.4, 22 °C).
2. A convenient purification of the oocyte homogenates is required.
3. For reactivation experiments, the oxime 2-PAM interferes with assay, and so must be removed after reactivation and before assay.
4. We wish to quantify the amount of each variant hBuChE as produced in oocytes.

To meet all these requirements, we have adapted a method of immobilizing the enzyme in monoclonal antibody-coated wells of microtiter plates. This method allows us to determine absolute amounts of bound hBuChE by an ELISA technique, K_i values, and rate constants for inhibition of the an enzyme by an OP and for the reactivation of the inhibited enzyme by 2-PAM (illustrated for reactivation experiments in FIG. 6).

TABLE 1. IC_{50} values (μ M) for hBuChE, hAChE and the hBuChE/hAChE chimera.

	eco-thiophate	BW284C51	isoOMPA	dibucaine	succinyl-choline	physo-stigmine	bambuterol
hBuChE	0.19	820	24	28	6500	0.29	0.31
chimera	0.023	26	450	260	3700	0.25	43
hAChE	0.028	0.18	330	990	1400	0.025	480

The concentration of inhibitor that results in 50% of the control rate (30 min, 22 °C) are presented.

Table 2. Substrate and inhibitor interactions with human butyrylcholinesterase

enzyme	relative K_m[a]	IC_{50}[b] (μM)		
		ecothiophate	*iso*-OMPA	DFP
wild-type[c]	1.0	0.27	22	0.02
$L^{286}K$	11	>1000	100	N.D.
$L^{286}Q$	9	5.5	140	0.25
$L^{286}R$	5	N.D.	N.D.	N.D.
$L^{286}D$	4	2.5	55	0.17
$F^{329}Q$	0.4	3.7	2	0.006
$F^{329}L$	0.8	2.5	4.8	0.004
$F^{329}C$	1.2	3.1	3	0.006
$F^{329}D$	1.0	2	30	0.03
$Y^{440}D$	2.1	0.68	85	0.07

[a]Ratios of K_m values toward butyrylthiocholine (wild-type = 1.0). Determinations were made on material from 3 or more different *in vitro* transcription and microinjection experiments with standard deviations.

[b]IC_{50} values are an average of 3 or more determinations of material from 2 or more microinjections. N.D., not determined; certain Leu^{286} mutants showed low activities, which made difficult precise determination of IC_{50} values.

[c]Oocyte activity following an injection of unmodified BuChEmRNA was used. This activity had K_m and IC_{50} values identical to those of human serum BuChE.

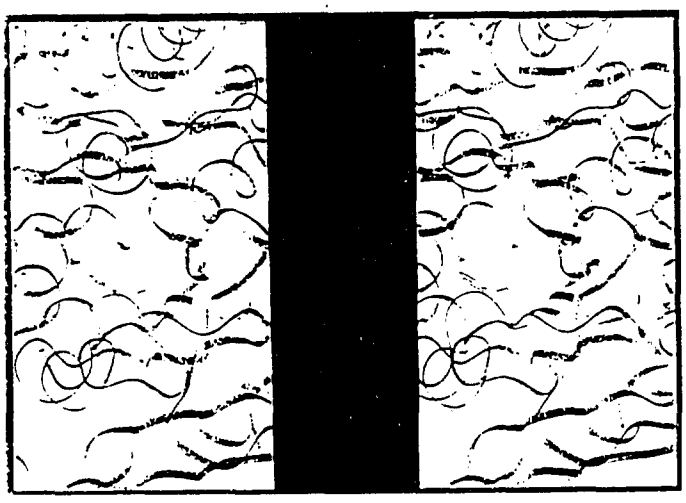

FIG. 3. Stereo view of the active site gorge of hBuChE. The backbone ribbon is colored as in FIG. 2. The catalytic triad residues, Ser^{198}, His^{438} and Glu^{325}, are shown in yellow; at the choline binding site Trp^{82} is green and Asp^{70} is red; at the acyl binding site Val^{288} and Leu^{286} are turquoise; at the peripheral anionic site Ala^{277} is black and Asn^{68} and Glu^{119} are orange.

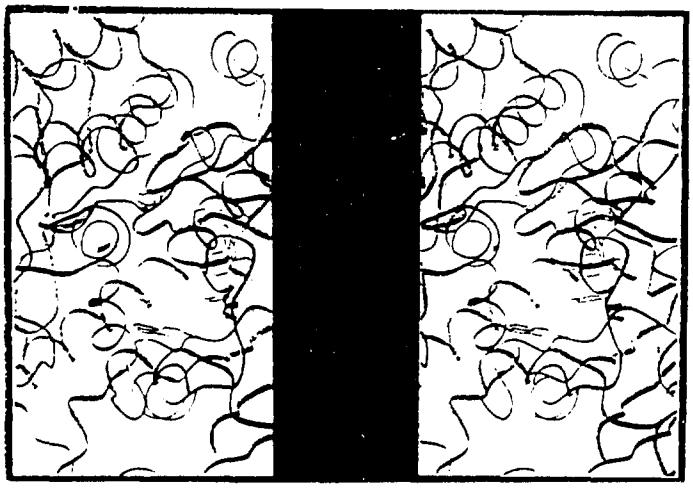

FIG. 4. Stereo view of the active site gorge of hAChE. Residues homologous with hBuChE are shown in the same colors as in FIG. 3.

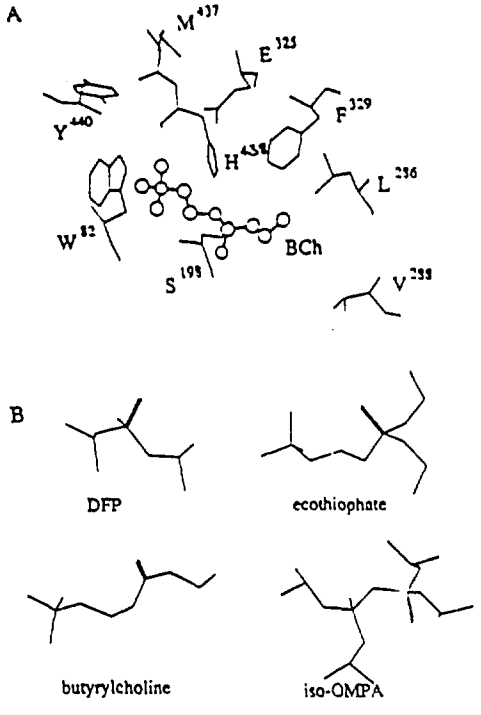

Fig. 5. **(A) The active site of hBuChE. Surrounded by several residues of the protein, a substrate molecule is shown in reaction with Ser[198] as its carbonyl carbon passes through a tetrahedral transition state.**

(B) Structures of selected organophosphorus anticholinesterases.

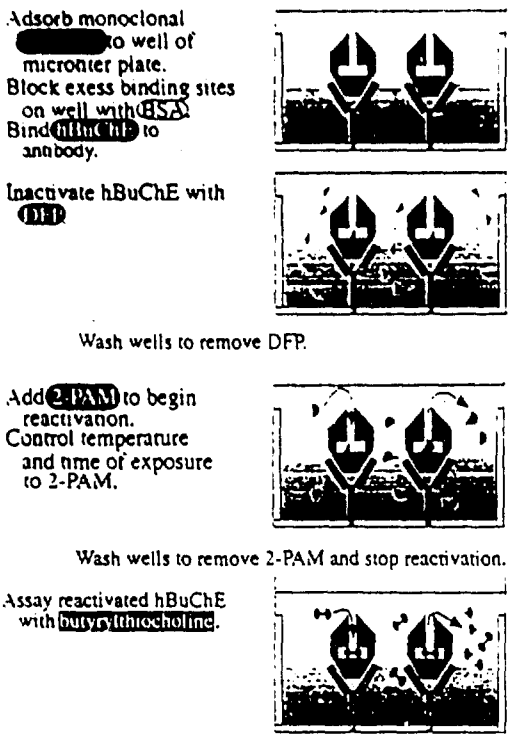

Fig. 6. Use of immobilized enzyme to observe kinetics of reactivation of OP-inhibited hBuChE.

PUBLICATIONS

Ben Aziz-Aloya R., Seidman S., Timberg R., Sternfeld M., Zakut H., and Soreq H. (1993) *Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of Xenopus embryos.* Proc. Natl. Acad. Sci. USA 90, 2471-2475.

Gnatt A., Loewenstein Y., Yaron A., Schwarz M., and Soreq H. (submitted for publication) *Site-directed mutagenesis of active site residues reveals plasticity of human butyrylcholinesterase in substrate and inhibitor interactions.*

Loewenstein Y., Gnatt A., Neville L.F., and Soreq H. (submitted for publication) *A chimeric human cholinesterase: identification of interaction sites responsible for recognition of acetyl- or butyrylcholinesterase-specific ligands.*

Neville L.F., Gnatt A., Loewenstein Y., Seidman S., Ehrlich G., and Soreq H. (1992) *Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE.* EMBO J. 11, 1641-1649.

Neville L.F., Gnatt A., Loewenstein Y., and Soreq H.(1990) *Aspartate-70 to glycine substitution confers resistance to naturally occurring and synthetic anionic-site ligands on in-ovo produced human butyrylcholinesterase.* J. Neurosci. Res. 27, 452-460.

Neville L.F., Gnatt A., Padan R., Seidman S., and Soreq H. (1990) *Anionic site interactions in human butyrylcholinesterase disrupted by two single point mutations.* J. Biol. Chem. 265, 20735-20738.

Prody C.A., Gnatt A., Zevin-Sonkin D., Gnatt A., Goldberg O., and Soreq H. (1987) *Isolation and characterization of full length cDNA clones coding for cholinesterase from fetal human tissues.* Proc. Natl. Acad Sci. USA 84, 3555-3559.

Soreq H., Gnatt A., Loewenstein Y., and Neville L.F. (1992) *Excavations into the active site-gorge of cholinesterases.* Trends Biochem. Sci. 17, 353-358.

Soreq H. and Zakut H. (1990) *Cholinesterase Genes: Multilevelled Regulation,* vol. 13 of Monographs in Human Genetics (Sparkes R.S. series ed.), pp. 1-108. Karger, Basel.